Molecular and morphometric variation in European populations of the articulate brachiopod *Terebratulina retusa*

B. L. Cohen [1], P. Balfe [1], M. Cohen [1], G. B. Curry [2]

[1] Department of Genetics, University of Glasgow, Glasgow G11 5JS, Scotland
[2] Department of Geology and Applied Geology, University of Glasgow, Glasgow G12 8QQ, Scotland

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Abstract. Molecular and morphometric variation within and between population samples of the articulate brachiopod *Terebratulina* spp., collected in 1985–1987 from a Norwegian fjord, sea-lochs and coastal sites in western Scotland, the southern English Channel (Brittany) and the western Mediterranean, were measured by the analysis of variation in the lengths of mitochondrial DNA (mtDNA) fragments produced by digestion with nine restriction endonucleases and by multivariate statistical analysis of six selected morphometric parameters. Nucleotide difference within each population sample was high. Nucleotide difference between population samples from the Scottish sites, both those that are tidally contiguous and those that appear to be geographically isolated, were not significantly different from zero. Nucleotide differences between the population samples from Norway, Brittany, Scotland and the western Mediterranean were also very low. Morphometric analysis confirmed the absence of substantial differentiation.

Introduction

Articulate brachiopods have short-lived, lecithotrophic larvae, a property that "should lead readily to spatial, reproductive and hence genetic isolation between ... populations ... the conditions under which speciation is most likely" (Rudwick 1970, see also Valentine and Jablonski 1983). This "divergence" hypothesis has never been tested by the study of genetic variability in geographically dispersed populations of articulate brachiopods. Accounts of genetic variability are limited to descriptions of allozyme variation in geographically undifferentiated deep-sea samples of *Freileia halli*, *Liothyrella notorcadensis* and *Coptothyris grayi* (Ayala et al. 1975, Valentine and Ayala 1975, Balakirev and Manchenko 1985) or of morphometric and molecular divergence between eastern and western North Atlantic morphospecies of *Terebratulina*, *T. retusa* and *T. septentrionalis* (Cohen et al. 1991). In the course of the latter study, we obtained population samples of the eastern Atlantic form, *T. retusa*, drawn from sites as far apart as Norway and the western Mediterranean, and this communication reports the results of analyses of these specimens for mitochondrial DNA (mtDNA) restriction-fragment length polymorphism (RFLP) and for variation in morphometric parameters of the shell.

For *Terebratulina* spp., the expectations of the divergence hypothesis are based on empirical and anecdotal evidence that larvae settle within 1 wk (Morse 1873, Noble et al. 1976, Webb et al. 1976). Support for the expectation that RFLP analysis of mtDNA would be capable of revealing such differentiation can be drawn from examples in which oceanographic or life-history factors provide mechanisms of isolation and where, using this methodology, 2% or more nucleotide divergence has been revealed between populations of marine organisms that are somewhat more vagile than articulate brachiopods (e.g. Avise et al. 1986, Baker et al. 1990, Reeb and Avise 1990). In contrast, and as expected, little or no divergence was found between highly vagile marine organisms (e.g. Graves et al. 1984, Wirgin et al. 1989).

Materials and methods

The numbers of individual specimens and geographic origins of the population samples of *Terebratulina retusa* are given in Table 1. Specimens from Tromsø, Firth of Lorne, Sound of Mull and the western Mediterranean were collected by dredge. All other specimens were hand-picked by SCUBA divers. The Scottish divers were requested to include all macroscopic articulate brachiopods from one or a few small areas of rock substrate. In practice, most diver-collected individuals were at least 1.0 cm long. Amongst dredged samples, individuals ≤ 0.6 cm, if collected, were not analysed. Thus, the analysis was restricted to brachiopods older than ~ 1 to 2 yr (Curry 1982). The Tromsø sample was suitable only for morphometric analysis.

Whilst most specimens were collected from closely circumscribed localities, those from the Mediterranean came from three separate collection sites. However, these sites are considered to be within the dispersal range of larvae (C. C. Emig personal communication 1990) and are closer together than the most distant pair of

Table 1. *Terebratulina retusa*. Geographical origin of population samples and specimen numbers analysed. Each population sample treated as a single operational taxonomic unit (OTU), except those indicated by asterisk, which constituted the OTU "Oban" (see "Results and discussion – *mtDNA analysis*")

Region Sampling-site	Longitude; Latitude	Numbers analysed for:	
		mtDNA	morpho-metrics
Norway			
Dröbak, (Oslo Fjord)	59′39″N; 10′48″E;	12	–
Tromsø	69′40″N; 19′00″E	–	17
Scotland			
Loch Duich	57′16″N; 05′31″W	10	–
Sound of Mull	56′31″N; 05′52″W	15*	15
Firth of Lorne	56′24″N; 05′33″W	19*	11
Luing (Balnahua, Culnach and Garvellachs)	56′16″N; 05′39″W	14*	–
Insh Island	56′18″N; 05′39″W	18*	–
Sound of Jura	56′05″N; 05′35″W	19*	–
Loch Fyne (Kenmore)	56′15″N; 05′59″W	16	21
Brittany (France)			
Isle de Batz (Roscoff)	48′44″N; 04′04″W	23	6
Western Mediterranean			
Calvi (Corsica)	42′42″N; 08′75″E	20	20
Isles d'Hyères	42′58″N; 06′24″E		
La Çiotat	42′59″N; 05′33″E		

Scottish sites. The Mediterranean specimens will therefore be treated as a single operational taxonomic unit (OTU).

Molecular and morphometric methods are described elsewhere in full (Cohen et al. 1991). Briefly, to estimate the nucleotide difference between mitochondrial DNA sequences, aliquots of total DNA were digested with nine informative restriction enzymes (*Acc*I, *Ava*I, *Ava*II, *Bgl*II, *Dra*I, *Eco*RV, *Pst*I, *Pvu*II, *Sst*I) and restriction fragments (>0.5 kbase pairs) were detected by agarose gel electrophoresis and capillary transfer to nylon membrane followed by hybridization with a radioactively-labelled, cloned, homologous mtDNA probe and autoradiography. The probe was a 15.8 to 16.5 kbase pair insert of quasi-complete *Terebratulina retusa* mtDNA in a lambda phage. Genetic distance (as nucleotide difference, $\hat{d}$, corrected for within-population divergence) was estimated from the frequencies of shared fragments and standard errors were obtained by 200 bootstrap cycles using the RESTSITE programs (Miller 1991). Since not more than 20 out of 179 (11%) restriction sites were mapped, $\hat{d}$ was estimated by the iteration procedure for unmapped fragments (Nei 1987: Eq. 5.54). We will refer to these estimates as $\hat{d}_f$. Nucleotide difference ($\hat{d}_s$) was also calculated by the "site" procedure (Nei and Li 1979, Nei and Miller 1990: Eq. 17) and both $\hat{d}_f$ and $\hat{d}_s$ values will generally be quoted in the expectation that the best estimate is intermediate. However, the restriction endonucleases used did not constitute a random sample; enzymes yielding <3 fragments in common mitotypes were considered uninformative and were not used. Thus, as is common in many such studies, $\hat{d}$ values are biased upwards. Cluster analysis of genetic distances was carried out by the UPGMA (unweighted pair group mean of analysis) procedure (Sokal and Michener 1958) using a program from the RESTSITE package, on the assumption that evolutionary rates in different lineages would be uniform. For morphometric analyses, six morphometric parameters, pre-selected by principal components analysis (PCA) to give maximum discrimination between brachiopod morphospecies, were measured by standard techniques. The parameters were: maximum length, width and height of conjoined valves, number of ribs in a 5 mm sector 10 mm anterior of the brachial valve umbo, lateral width of the pedicle foramen, and anterior-posterior width of the pedicle foramen. The data were analysed by standard statistical procedures including PCA.

Results and discussion

mtDNA analysis

Results with all nine restriction endonucleases were obtained on DNAs from 109 individuals of *Terebratulina retusa*, amongst which 84 different nine-enzyme haplotypes ("mitotypes") were identified. These mitotypes and their geographic distribution are given in Table 2. Results with 5 to 8 enzymes were obtained on a further 57 individuals, so that the total number of individuals analysed was 166. The number of different restriction fragments identified was 170 and the average number of fragments per individual was ~42. Thus, ~240 bp (1.5%) of the mitochondrial genome was screened for variation. The relatively low resolution of this analysis was dictated by limiting yields of DNA, reflecting the low biomass of the smaller individuals of *T. retusa*. The average nucleotide difference between all pairs of individuals ($n=166$) was $d_f=0.0212\pm0.0067$, $d_s=0.0577\pm0.0170$.

Length variation greater than ~150 bp was not detected in the mtDNA of *Terebratulina retusa* using both a sensitive test with an internal standard and cross-comparison of digests, whereas such variation was ubiquitous in the western Atlantic morphospecies *T. septentrionalis*. All variants were therefore treated as changes in restriction sites and included in the analysis. Heteroplasmy was also not detected in *T. retusa* although it was found in *T. septentrionalis* (Cohen et al. 1991).

mtDNA diversity in *Terebratulina retusa*

Nucleon (haplotype) diversity was calculated from the frequencies of the 84 different mitochondrial haplotypes recognized amongst the 109 individuals that were analysed successfully with all 9 restriction enzymes (Table 2). The resulting value was $\hat{h}=0.9886$. Similar high values were obtained in each population sample except that from Dröbak, where $\hat{h}=0.85$ (see legend to Table 3). The geographic distribution of the mitotypes is given in Table 2.

Haplotype diversity values as high as 0.99 have rarely been observed in other organisms and have been given special attention because of the possibility of characterising individuals by their mitotype ("fingerprinting", Avise et al. 1989). In *Terebratulina retusa*, these high values were obtained with a relatively small number of restriction digests. With a more sensitive assay (Wilson et al. 1985), almost every individual would be readily distinguishable. Thus, haplotype diversity assays are somewhat artificial, being strongly affected by the number of restriction endonucleases used and the extent to which they are selected for informativeness. Nevertheless, it is clear that many organisms (especially vertebrates) show far

Table 2. *Terebratulina retusa*. Geographic distribution of nine-enzyme mitotypes amongst OTUs (see Table 1). mtDNA morphs recognized for each enzyme are identified alphabetically, but for restriction enzyme *Ava*II, more than 26 morphs were identified and those following "Z" are identified by numbers. Mitotypes are arranged from the left to right in alphabetical order of restriction enzymes, *Acc*I, *Ava*I, *Ava*II, *Bgl*II, *Dra*I, *Eco*RV, *Pst*I, *Pvu*II, *Sst*I. Hence, mitotype "AAAABCAAD" indicates presence of "A" morphs for *Acc*I, *Ava*I, *Ava*II and *Bgl*II, "B" morph for *Dra*I, "C" morph for *Eco*RV, etc. Note that there are no entries in this Table for individuals from Dröbak OTU, from which only incomplete, 5- to 8-enzyme mitotypes were obtained

Mitotype	OTU					
	Loch Duich	"Oban"	Loch Fyne	Isle de Batz	Mediterranean	Total
AAAABCAAD		1				1
AADAAAAAA	1	1				2
AADABAAAA		5				5
AADBCAAHA					1	1
AAEABAAAD			1			1
AAEABCAAB	1	2	1	1		5
AAEBCCAAB		1				1
AAFABAAAD		1				1
AAGABAAAA		1				1
AAHABAAAF		1				1
AAHABCAEG				1		1
AAIABAAAA		1				1
AAUAAAAAA			1			1
AAWABBAAA		1				1
AA2AHAAAA					1	1
AA3ABAAAF					1	1
AA7ABCAAG	1					1
AA9IBCAAA			1			1
ABAABAAAA		1				1
ABDABAAAA		1				1
ABDABAAAB		1				1
ABDABAAAF		1				1
ABIAAAAAA		1				1
ABIABAAAA	1	4	2	2		9
ABIABAAAE		1				1
ABIABAAAF		2				2
ABIABAAAG		1				1
ABIABAACA		1				1
ABIABAACF		1				1
ABIABCACA		1	1			2
ABIACAAAA		1				1
ABIACAACF				1		1
ABIACAAGA		1				1
ABIACCAAA				1		1
ABIAEAAAA		1				1
ABIAHAAAA		1				1
ABIBBAAAA		3				3
ABPABAAAA		1				1
ABRABAAAD		1				1
ABRABAACA		1				1
ABRACAAAA				1		1
ABXACAAAA				1		1
AB6ABAAAA		1				1
ACDABAABA		1				1
ACFAAAAAD		1				1
ACFABAAAD			1			1
ACHABCAAA		1				1
ACIABAAAA		1				1
ACIABAAAF		1				1
ACVABAOAA		1				1
AC2ACAABA		1				1
AHYABAAAA	1					1

Table 2 (continued)

Mitotype	OTU					
	Loch Duich	"Oban"	Loch Fyne	Isle de Batz	Mediterranean	Total
AIDAAAAAA			1			1
AIDABAAAA	2	1				3
AIDMBAAAA	1					1
AIVAAAAAA	1					1
AJDABAAAA		1				1
AJGABCAAA		1	1			2
AJHAACAAD		1				1
AJKABCAAD		1				1
AJKEBCAAA		1		1		2
AKCABAAAA		1				1
AKCABAAAD		1				1
AKIABAAAA		1				1
ALIAHACAA				1		1
APDABAAAA	1					1
ASDAAAAAA				1		1
BCHABCAAA			1			1
BCHACAADA				1		1
BCKABCAAA		1				1
DDJABCAAA				1		1
DDOAACAAA		1				1
DOIKCAACA		1				1
DQJAACAAA		1				1
EADABCAAA					1	1
GADABAAAB				1		1
GRZDAAAAG				1		1
HD8NIAAAA			1			1
IAAABAAAA		1				1
JBAABAAAA		1				1
JB5ABAAAD					1	1
JDPABAAAA				1		1
LBAAFAAAA		1				1
NCHABCAAA		1				1
Totals	10	66	12	16	5	109

lower levels of diversity than *T. retusa*, such that within any population many individuals possess the same mitotype when analysed using a similar number of enzymes.

High levels of haplotype diversity are reflected by high within-population nucleotide divergences: (mean $\pm$ SD, $n=10$) $\hat{d}_f = 0.0182 \pm 0.0042$ (range 0.0105 to 0.0258); $\hat{d}_s = 0.0624 \pm 0.0123$ (range 0.0309 to 0.0757). Such diversity may result from a high rate of mtDNA evolution and/or large (stable or expanding) effective population size (Avise et al. 1984). For the reasons given below, we identify large or growing effective population-size as the most important factor in *Terebratulina retusa*.

The rate of mtDNA evolution in *Terebratulina retusa* and *T. septentrionalis* can be roughly estimated as follows. We have reported (Cohen et al. 1991) mtDNA nucleotide difference between the western and eastern North Atlantic species *T. septentrionalis* from Canada ($n=40$) and *T. retusa* from Scotland ($n=56$). Recalculated with the procedures used in the present report these values are $\hat{d}_f = 0.1215 \pm 0.0572$, $\hat{d}_s = 0.3493 \pm 0.0760$. If the onset of complete geographic isolation between these species occurred during the opening of the North Atlantic, this level of divergence must have accumulated during some

50×10^6 yr, indicating an evolution rate of 0.001 to 0.0035 substitutions per nucleotide per million years per lineage. This very approximate estimate suggests that the rate of mtDNA evolution in these *Terebratulina* species is not abnormally high, but might be unusually low (Vawter and Brown 1986, Jacobs 1988).

Several lines of evidence indicate that effective population sizes may be large (i.e., considerably greater than 10^4). These include: (1) diver's reports (e.g. A. S. G. Curtis personal communication) that the Insh Island, Sound of Jura and Loch Fyne populations, whilst patchy, may reach densities of many hundreds of individuals per square metre over extensive areas, indicative of census sizes of the order of at least 10^5 to 10^6; (2) generation time may be extrapolated from data in Curry (1982) to be at least 3 to 5 yr; (3) the secondary sex ratio, determined by microscopic observation of gonads, is statistically indistinguishable from 1:1 (BLC and MC unpublished observations); (4) during the breeding season, ripe gonad was present in every individual of the SCUBA-collected samples over a minimum size probably corresponding to the second year class (BLC and MC unpublished observations) and (5) there is no reason to suppose adverse environmental conditions since a marine environment returned to the sites sampled.

Genetic divergence between geographically isolated sampling sites

mtDNA analysis

Some of the Scottish sampling sites (in north-south order Sound of Mull, Firth of Lorne, Luing, Insh Island and Sound of Jura) were sampled from a contiguous 80 km stretch of the west coast of Scotland (Fig. 1) where tidal currents are of the order of 1 m/s and longshore currents are common (Allen et al. 1986). We therefore anticipated that the brachiopod populations at these sites would not be effectively isolated from one another, except that the Sound of Mull and Firth of Lorne samples, which were dredged from ~200 m, might prove to be isolated (by the thermocline) from the other populations, which were collected by SCUBA divers from depths between ~25 and 40 m. The results support the expectation of no divergence: none of the pairwise nucleotide differences was significantly different from zero (largest $\hat{d}_f = 0.0001 \pm 0.0003$; largest $\hat{d}_s = 0.0002 \pm 0.0007$). Thus, isolation by the thermocline was not detected. The specimens from all these sites were therefore pooled for further analysis to form a single OTU "Oban".

The remaining two Scottish samples (Loch Duich and Loch Fyne) represent populations from sea-lochs situated respectively ~100 km north and ~250 km south and east (by sea) of the "Oban" locality, separated from it by a dissected coastline (Fig. 1) and, at least in the case of Loch Fyne, by tidal streams and land-forms that permit little inter-communication (Allen et al. 1986). They are thus likely to be geographically isolated from the sites of the "Oban" OTU and from one another and are treated separately throughout. Nevertheless, genetic distances

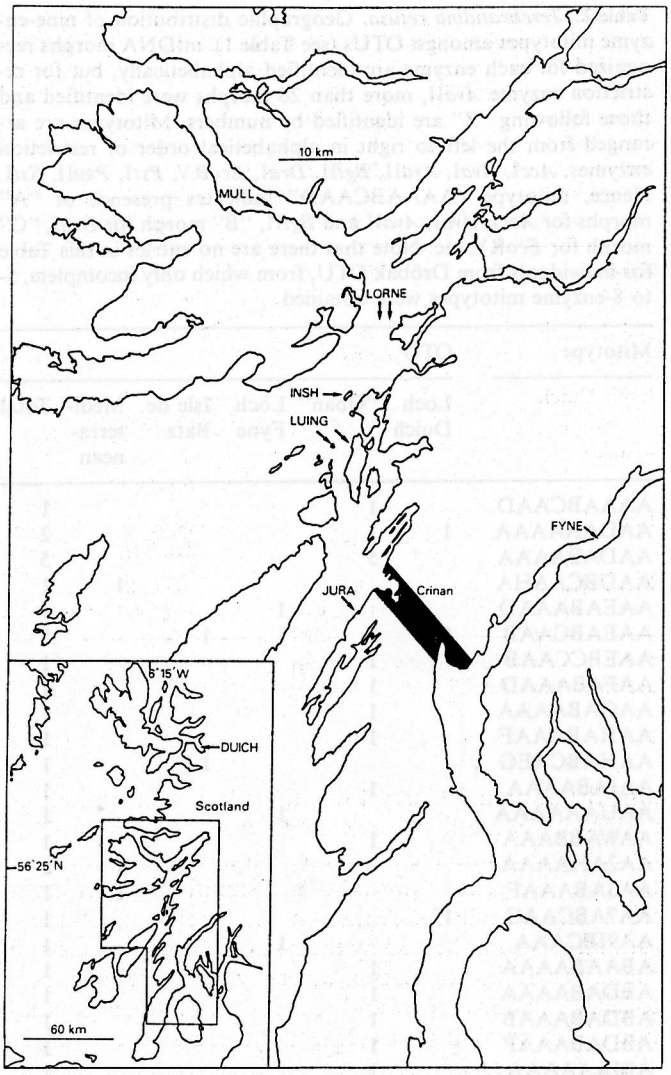

Fig. 1. Scottish collection sites; inset shows small-scale locality-map with area of larger-scale map outlined. General locality of former sea-way at Crinan is shaded, areas from which samples were collected are arrowed. Full details of collection sites are given in Table 1

are not significantly different from zero (Table 3). There is particularly good historical reason for anticipating genetic continuity between the "Oban" and Loch Fyne samples: the Sound of Jura and Loch Fyne were connected by a sea-way at Crinan (Fig. 1) until isostatic uplift (ca. 10 000 yr B.P.) following retreat of the ice-cap (Peacock et al. 1977). Thus there has been little time for divergence between the "Oban" and Loch Fyne OTUs.

The sampling localities in Norway, Scotland, Brittany and the western Mediterranean are on the order of 1 to 10×10^3 km apart. Considering the patchy distribution and limited dispersal potential of *Terebratulina retusa*, it may be strongly conjectured that these distant sites are effectively isolated from one another. This suggestion is supported by distribution data: *T. retusa* is absent from the Plymouth Marine Laboratory's collecting area in south-western England (Marine Biological Association 1957) and it has not been reported to us by divers searching between Cornwall and the east coast of Scotland (see

Table 3. *Terebratulina retusa.* Net mtDNA divergence between population samples. Below diagonal: nucleotide difference estimates ($\hat{d}_f$) derived by "fragment" procedure. Above diagonal: nucleotide difference estimates ($\hat{d}_s$) derived by "sites" procedure. Standard errors are given in parentheses. Asterisks indicate nucleotide difference estimates significantly different from zero (+2 SE). "Oban" includes data for Sound of Mull, Firth of Lorne, Luing, Insh Island and Sound of Jura (see Table 1). Effect of low DNA yields from Drobak specimens was assessed by replacing missing digests (1 out of 12 *Acc*I, all of 12 *Ava*I and 6 of 12 *Ava*II) with data selected (by random numbers, with replacement) from mitotypes of all 109 fully classified individuals. RESTSITE analysis indicated that missing data had no important effect on overall result; Mediterranean sample was similarly affected, but to lesser extent

	Drobak	Isle de Batz	Loch Duich	"Oban"	Loch Fyne	Mediterranean
Drobak		0.0150 (0.0136)	0.0210 (0.0164)	0.0166 (0.0155)	0.0206 (0.0164)	0.0214 (0.019)
Isle de Batz	0.0052 (0.0054)		0.0015 (0.0012)	0.0004 (undef.)[a]	−0.0004 (undef.)[a]	0.0024* (0.0010)
Loch Duich	0.0073 (0.0065)	0.0005 (0.0004)		0.0039 (0.0028)	−0.0002 (undef.)[a]	0.0040* (0.0019)
"Oban"	0.0057 (0.0046)	0.0000 (undef.)[a]	0.0013 (0.0011)		0.0003 (undef.)[a]	0.0054* (0.0026)
Loch Fyne	0.0071 (0.0071)	−0.0002 (undef.)[a]	−0.0001 (undef.)[a]	0.0000 (undef.)[a]		0.0021* (0.0008)
Mediterranean	0.0074 (0.0072)	0.0008 (0.0004)	0.0013 (0.0007)	0.0019* (0.0009)	0.0007* (0.0002)	

[a] Negative $\hat{d}$ values arise when mean within-population nucleotide difference is greater than between-population $\hat{d}$ value. Standard errors are then "undefined"

also Brunton and Curry 1979, their Fig. 7). Despite this apparent geographic isolation between Norway, Scotland, Brittany and the Mediterranean, only a minority of the pairwise genetic distances are significantly different from zero (Table 3). Although interpretation of these estimates is complicated by their known upward bias and the high within-population diversity (which contributes to large standard errors and reduces net between-population differences), it seems clear that there is little evidence of genetic differentiation between these populations. This conclusion is supported by the geographic distribution of mitotypes (Table 2), where the principal indication of geographic differentiation lies in the frequencies of "endemic" mitotypes, i.e., those found in only one sample. These frequencies are: Loch Duich, none out of 10; Loch Fyne, 7 of 12; "Oban" 48 of 66, Brittany, 5 of 16; Mediterranean, 5 of 5; i.e., there is a weak indication of endemicity in the Mediterranean sample. UPGMA cluster analysis of the $\hat{d}_f$ values between each pair of the 84 mitotypes, values that are not affected by within-sample diversity, confirmed this conclusion (analysis not shown). The hypothesis that there is covariance of genetic and geographic distances was also excluded by a Mantel test (Manly 1985, analysis not shown). Thus it seems that the (mitochondrial) genomes of geographically isolated populations of *Terebratulina retusa* from Norway to the Mediterranean are barely differentiated, contrary to expectation under the divergence hypothesis.

Morphometric analysis

Amongst the six morphometric parameters analysed on shells from six population samples, the only significant difference was in shell length between the Loch Fyne and

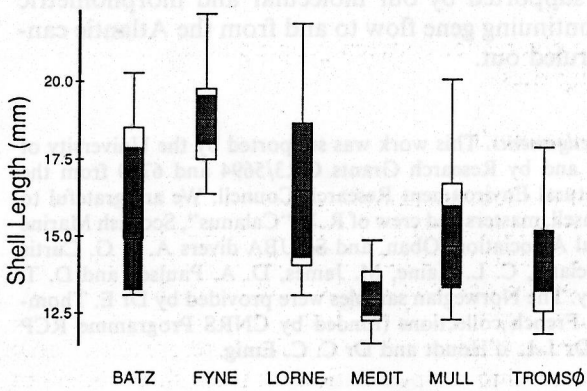

Fig. 2. *Terebratulina retusa.* Boxplots of shell lengths. Shaded areas indicate 95% confidence limits around median values. Numbers of shells measured: Tromsø, 17; Sound of Mull, 15; Firth of Lorne, 11; Loch Fyne, 21 Isle de Batz, 6; Mediterranean, 20

Mediterranean samples (Fig. 2). In the principal components analysis on these data, the first three axes accounted for 90.2% of the total variance and no clear between-population discrimination was found except for some separation on the first axis between the Loch Fyne and Mediterranean samples. In a previous brachiopod study, first axis discrimination was associated with size variation (McCammon and Buchsbaum 1968), corresponding to the shell-length difference shown in Fig. 2. Apart from this size difference, the populations sampled were not morphometrically distinguishable. A similar result has been obtained on many other samples (totalling over 1000 specimens) of *Terebratulina retusa* throughout its European range (Curry unpublished data). Thus, there is neither morphometric nor genetic evidence for cryptic speciation in the samples examined.

History of the populations sampled

The sampled populations of *Terebratulina retusa* occupy sites close to or above the 200 m depth contour and must therefore have been established not more than ~10 000 yr B.P., as the sea level rose (Milliman and Emery 1968, Morner 1982). The low between-population divergence now observed is consistent with the hypothesis that the ancestral population was large, widespread and highly diverse and that little or no genetic diversity was lost as its range extended (Avise et al. 1984). Alternatively, larvae from genetically divergent ancestral populations may have intermingled during range extension, perhaps influenced by the North Atlantic current (Curry and Endo 1991).

The suggestion that Recent *Terebratulina retusa* populations in the western Mediterranean were established during the 200 m sea-level rise is in agreement with the hydrography of the western Mediterranean (Margalef 1985) and with the affinities of other Mediterranean invertebrates (Logan 1979).

Davidson (1886–1888: footnote p. 25/26) may have accepted Mediterranean *Terebratulina retusa* as a distinct variety or subspecies (*emarginata*), whilst Logan (1979) followed Dall (1920) in rejecting this. Dall's approach is clearly supported by our molecular and morphometric data; continuing gene flow to and from the Atlantic cannot be ruled out.

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